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ORIGINAL PAPER

Post-transcriptional gene silencing and virus resistance in *Nicotiana benthamiana* expressing a *Grapevine virus A* minireplicon

Marina Brumin · Svetlana Stukalov · Sabrina Haviv · Mookkan Muruganantham · Yoni Moskovitz · Ozgur Batuman · Annie Fenigstein · Munir Mawassi

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Abstract *Grapevine virus A* (GVA) is closely associated with the economically important rugose-wood disease of grapevine. In an attempt to develop GVA resistance, we made a GFP-tagged GVA-minireplicon and utilized it as a tool to consistently activate RNA silencing. Launching the GVA-minireplicon by agroinfiltration delivery resulted in a strong RNA silencing response. In light of this finding, we produced transgenic *Nicotiana benthamiana* plants expressing the GVA-minireplicon, which displayed phenotypes that could be attributed to reproducibly and consistently activate post-transcriptional gene silencing (PTGS). These included: (i) low accumulation of the minireplicon-derived transgene; (ii) low GFP expression that was increased upon agroinfiltration delivery of viral suppressors of silencing; and (iii) resistance against GVA infection, which was found in 60%, and in 90–95%, of T1 and T2 progenies, respectively. A grafting assay revealed that non-silenced scions exhibited GVA resistance when they were grafted onto silenced rootstocks, suggesting transmission of RNA silencing from silenced rootstocks to non-silenced scions. Despite being extremely resistant to GVA infection, the transgenic plants were susceptible to the closely related vitivirus, GVB. Furthermore, infection of the silenced plants with GVB or *Potato virus Y* (PVY) resulted in suppression of the GVA-specific defense. From these data we conclude that GVA-minireplicon-mediated RNA silencing provides an important and efficient approach for consistent activation of PTGS that can be used for controlling grapevine viruses. However, application of this strategy for virus resistance necessitates consideration of possible infection by other viruses.

Keywords Vitiviruses · RNA silencing · Suppression of gene silencing · Genetically engineered virus resistance · Grafting

M. Brumin · S. Stukalov · S. Haviv · M. Muruganantham · Y. Moskovitz · O. Batuman · A. Fenigstein · M. Mawassi (✉)
The S. Tolkowsky Laboratory, Department of Plant Pathology-The Virology Unit, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel
e-mail: mawassi@volcani.agri.gov.il

Introduction

Grapevine virus A (GVA) is closely associated with the economically important rugose-wood (RW) disease of grapevine, specifically with the Kober stem grooving (Martelli 1993; Garau et al. 1994; Chevalier et al. 1995). The virus is spread with infected propagation plant material and through transmission by mealybugs. It has elongated flexuous particles about 800 nm long and is considered to be a phloem-associated pathogen (Minafra et al. 1994, 1997). Some isolates of GVA have been mechanically

transmitted to herbaceous plants (Monette and James 1990). So far, the use of virus-free propagation plant materials is the strategy employed for controlling GVA, as well as other grapevine viruses.

GVA, as well as the serologically distinct virus *Grapevine virus B* (GVB), are members of the genus *Vitivirus*, family *Flexiviridae* (Martelli et al. 2007). The virus particle contains a positive single-stranded RNA genome of ca. 7.4 kb, which consists of five open reading frames (ORFs) (Minafra et al. 1994, 1997). ORF1 encodes a 194-kDa polypeptide with characteristics of replication-associated proteins. ORF2 encodes a 19-kDa protein with no significant similarity to other proteins. ORF3 encodes a 31-kDa viral movement protein. ORF4 encodes the coat protein (CP). ORF5 encodes a 10-kDa protein (p10) that exhibits sequence similarities to small RNA-binding proteins of various plant viruses (Galiakparov et al. 2003b). Recently, p10 was reported to possess an RNA-silencing suppressor activity (Chiba et al. 2006; Zhou et al. 2006). The products of ORFs 2–5 are dispensable for replication in protoplasts (Galiakparov et al. 2003a, c). GVA-infected cells contain, in addition to the full-length genomic RNA, two nested sets of sgRNAs: one comprising at least three 5′-terminal molecules and the other comprising at least three 3′-terminal sgRNA molecules (Galiakparov et al. 2003a). The production of the 5′- and 3′-terminal sgRNAs has been hypothesized to be controlled by sequences upstream of the 5′-terminus of each of ORFs 2–4 (Galiakparov et al. 2003a).

Development of virus-resistant plants, which involves their genetic transformation, is considered to be a promising means for reducing virus damage. In the process of constructing such resistant plants, a transgene derived from the viral genome is incorporated into the plant genome. Earlier examples of pathogen-derived resistance were dependent upon the expression and accumulation of viral proteins in transgenic plants (Powell et al. 1990). Thus, using *Nicotiana benthamiana* as a model system, transgenic plants were obtained by transformation with the genes of GVA and GVB that encoded the coat- and movement proteins (Minafra et al. 1998; Radian-Sade et al. 2000). However, the resulting plants, were not sufficiently resistant to virus infection, and exhibited delayed virus accumulation. Later studies revealed that imparting resistance does not necessary require accumulation of the viral proteins, but could be achieved with constructs designed to generate RNA that is not translatable (de Haan et al. 1992; Lindbo and Dougherty 1992; Baulcombe 1996). This type of RNA-mediated resistance, which appears to induce a form of post-transcriptional gene silencing (PTGS) response, has proved to be very robust in protecting plants from infection by high concentrations of viruses (Baulcombe 1996, 1999).

At the heart of PTGS stands the double-stranded RNA (dsRNA). The dsRNA is recognized and cleaved into small interfering RNAs (siRNAs) of 20–24 nucleotides by an RNase III-like enzyme named DICER (Fire et al. 1998; Bernstein et al. 2001; Hamilton et al. 2002; Deleris et al. 2006). The siRNA guides a multi-subunit ribonuclease, named RNA-induced silencing complex (RISC), and ensures specific degradation of RNAs that share sequence similarity with the dsRNA (Bernstein et al. 2001; Hammond et al. 2001). It was hypothesized that siRNAs spread in plants from cell to cell through plasmodesmata and are transported in the phloem, and thereby trigger silencing throughout the plant (Palauqui et al. 1997; Voinnet et al. 1998).

Although antiviral protection by means of plant transformation was found to be successful, this approach does not always result in efficient expression of the transgene, and effective PTGS. Transgenes that were designed to express RNAs with a self-complementary sequence (hairpin RNA [hpRNA]) appeared to be more efficient in inducing PTGS than the conventional sense or antisense viral transgenes (Wang et al. 2000). Furthermore, there are often variable expression levels of the transgene, which are frequently affected by position effects, chromatin effects, T-DNA integration patterns, and transgene copy number (Lewin 1994; Mlynarova et al. 1995; Allen et al. 1996). In order to overcome such chromosomal effects and to reduce the between-line variation, Angell and Baulcombe (1997, 1999) as well as Dalmay et al. (2000) developed transgenic Arabidopsis plants transformed with a cDNA of replicating RNAs of *Potato virus X* (PVX), which includes the PVX sequences of RdRp, the "Triple gene block" and the CP. The expressed PVX amplicons were found to be efficient activators of PTGS, which provided an important new strategy for the consistent activation of PTGS in transgenic plants. On the basis of this strategy, in the present study we investigated the PTGS phenotype of

expressing replicating RNA of the GVA in *N. benthamiana* plants. We developed *N. benthamiana* plants expressing a GFP-tagged GVA minireplicon that expressed the products required for RNA replication, but did not express either the movement- and coat proteins or the suppressor of RNA silencing, p10. The GVA-minireplicon was found to be an efficient inducer of the PTGS response and its expression in transgenic plants resulted in highly effective GVA-specific resistance.

Materials and methods

Construction of the GFP-tagged GVA-minireplicon

The clone pGR5 (Galiakparov et al. 2003a), with the full-length infectious GVA cDNA (GenBank accession number AY244516) cloned into pUC57 (MBI Fermentas, Canada) under the T7 RNA promoter, was used for the subsequent experiments in the present study. PCRs used for this study were carried out with the PfuTurbo (Stratagene) DNA polymerase enzyme.

The sequence of the eGFP gene, with the *Not I* cleavage site downstream of its stop codon, was fused to the end of ORF2 of GR5 by means of overlap extension PCR products (Ho et al. 1989). The resultant construct was next cleaved with *Not I* and *BssH II* (cleaves ORF5 at downstream position 50), followed by filling and ligation of the ends to generate pGVA-GFP-260. Thus, the cDNA of GVA-GFP-260 was composed of the 5′-UTR, ORF1, ORF2 and the 3′-290 nts and the eGFP gene fused to the 3′-end of ORF2.

For transformation or agro-inoculation, the entire cDNA of GVA-GFP-260 was amplified from pGVA-GFP-260 by *Pfu* DNA polymerase (Finnzymes, Finland) and inserted between the 35S promoter and terminator derived from *Cauliflower mosaic virus* (CaMV) of the binary vector pCAMP-35S (Moskovitz et al. 2008) cleaved at the sites of *Stu* I and *Sal* I to generate pGVA-GFP-269.

Protoplast isolation and inoculation

Protoplasts were isolated from fully expanded leaves of *N. benthamiana* and transfected with RNA transcribed from the pGVA-GFP-260 as described previously (Galiakparov et al. 2003a; Haviv et al. 2006).

Agroinfiltration

Agroinfiltration on *N. benthamiana* leaves was carried out according to Johansen and Carrington (2001), using *Agrobacterium tumefaciens* strain EHA 105 that contains a binary vector carrying pGVA-GFP-269, which does not express a viral suppressor of RNA silencing, together with an *Agrobacterium* that contains a binary vector carrying a candidate suppressor gene. The cDNA of the RNA silencing suppressor of GVA (p10), cloned into the binary vector pCAMP-35S (Moskovitz et al. 2008), as well as clones expressing the suppressors of RNA silencing derived from, PVX p25, TBSV p19, ZYMV Hc-pro and TYLCV V2 were used in this study.

Plant transformation and analysis

The binary plasmid carrying the mini-replicon of GVA (pGVA-GFP-269) was transformed into *A. tumefaciens* strain EHA 105. Transgenic *N. benthamiana* plants were obtained as described by Horsch et al. (1985). Plantlets were confirmed as transgenic according to their resistance to kanamycin, as well as the results of GUS, PCR, Southern blot and confocal microscopy assays.

DNA extraction, Southern blot and PCR analyses

Genomic DNA was extracted from leaves as described by Doyle and Doyle (1987). Fifteen micrograms of gDNA were digested overnight by *Hin*dIII, separated on 0.9% (w/v) agarose gel and blotted onto positively charged nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization of the membrane was carried out at 42°C with a Digoxigenin (DIG)-labeled riboprobe (Roche Molecular Biochemicals) specific to the 5′-1-kb of the GVA genome, according to the manufacturer's instructions. Digestion of the DNA of transgenic plants by *Hin*dIII was expected to result on a 2-kbp fragment, because of the presence of two *Hin*dIII cleavage sites: one in the binary vector and the other in transgene GVA-GFP-269. For PCR, a fragment that covered about 1.1 kb of the fused

ORF2 and eGFP sequences was amplified with the primer combination GVA14 (5′-CCTAGGATATCA-GAGTATAGATAGCAT-3′) and GVA125 (5′-TTA CTTGTACAGCTCGTCC-3′).

RNA isolation and northern blot analyses

Double-stranded RNAs were extracted from *N. benthamiana* protoplasts as described by Galiakparov et al. (2003a). Total RNA of plants was extracted from the young leaf tissues using Tri Reagent (Sigma–Aldrich, USA), according to the manufacturer's instructions. Five micrograms of total RNA were separated in 1.2% formaldehyde-agarose gel. Northern blotting and hybridization for detection of GVA RNA were conducted as described previously (Galiakparov et al. 2003a; Haviv et al. 2006), with a DIG-labeled riboprobe specific to the 5′-1.0 kb of the GVA genome.

For siRNA analysis, 15–20 μg of the total RNA were separated on 15% (w/v) polyacrylamide gel containing 7 M urea and electrotransferred to a positively charged nylon membrane (Roche Molecular Biochemicals). The blots were hybridized at 37°C with alkaline-hydrolyzed DIG-labeled RNA probe. For alkaline hydrolysis, the riboprobe was incubated with carbonate buffer (60 mM Na_2CO_3, 40 mM $NaHCO_3$, pH 10.2) at 60°C for 1 h, followed by addition of neutralization buffer (200 mM sodium acetate, 1% (v/v) acetic acid, pH 6.0) and RNA precipitation.

GFP imaging

GFP expression in infiltrated leaves or in putative transgenic plants was monitored with the OLYMPUS confocal laser-scanning microscope. For GFP quantification, image analysis of average fluorescence intensity per square micrometer was carried out with the Multi-image Quantification Analysis (MICA) system (Cytoview Ltd, Petach Tikva, Israel). The statistical difference in average area intensity in the different infiltrated areas of leaves was calculated by analysis of variance (ANOVA) with Microsoft Excel software (Microsoft, Redmond, WA). Quantitative analysis of the fluorescence intensity per unit area was obtained from five different regions of infiltrated leaves (five images per leaf).

Virus source, infection and virus resistance assay

Non-transgenic *N. benthamiana* plants were inoculated with in vitro RNA transcripts prepared from the full-length infectious clones of GVA (Galiakparov et al. 2003a) or GVB (Moskovitz et al. 2008). For further plant inoculation, symptomatic leaves of GVA- or GVB-infected plants (10–15 days post infection (dpi)) were ground in 0.1 M phosphate buffer (pH 7.0) and the sap was used as a virus inoculum to inoculate leaves of transgenic or non-transgenic *N. benthamiana* plants dusted lightly with carborundum. Symptoms of GVA infection were monitored 6–15 dpi for non-transgenic plans and up to 90 dpi for transgenic plants. *Potato Virus Y* (PVY) was propagated in *N. benthamiana* plants, which similarly were used as a source for sap preparation.

Grafting technique

Only plants that were confirmed to be transgenic by confocal microscopy have been chosen for cleft grafting as described by Palauqui et al. (1997). Briefly, the rootstocks were beheaded 10 cm above the soil, and a vertical cut of ~1–1.5 cm was made in the center of the stem. The terminal apex of the plant to be used as a scion was excised and beveled, and its leaves were removed. The rootstock and the scion were fastened together with Parafilm. During the first week after grafting, the scion was covered with a plastic bag to prevent dehydration.

Results

Replication assay of GVA minireplicon

Previously, we reported that GVA replicons containing ORF1 and the terminal UTRs of the GVA genome were capable of replication (Galiakparov et al. 2003a), indicating that the GVA ORFs 2–5 were dispensable for virus replication. In light of those findings, a GFP-tagged GVA construct, designated as GVA-GFP-260, was assembled in pUC57 (MBI Fermentas) under the T7 RNA promoter (Fig. 1a). The RNA transcribed from pGVA-GFP-260 was examined in *N. benthamiana* protoplasts. Hybridization analysis of RNA extracted from GVA-GFP-260-infected protoplasts with a 5′-specific RNA probe

showed the genomic RNA and a 5′-terminal sgRNA (Fig. 1b), which probably is generated via controller elements located upstream of ORF2, as previously reported by Galiakparov et al. (2003a). Moreover, the 5′-specific RNA riboprobe reacted with a small RNA of ca. 150 nts (Fig. 1b). The origin of this small RNA remains to be determined; it is most likely associated with GVA infection (Mawassi 2007). Protoplasts infected with GVA-GFP-260 were examined also by means of confocal microscopy. Green fluorescence was detected in 20–30% of the protoplasts (Fig. 1c). Taken together, these results indicated that the GVA-GFP-260 was able to replicate and to express ORF2:GFP sgRNA. In light of these findings, GVA-GFP-260 and its derivative GVA-GFP-269 (see below), will be described here as GVA-minireplicons.

GVA replication and expression of the viral sgRNA is known to be associated with generation of dsRNA (Galiakparov et al. 2003a). To examine whether GVA-GFP-260 minireplicon-infected cells contained these forms of RNA, dsRNA was extracted from inoculated protoplasts and subjected to northern blot analyses, with plus- and minus-specific riboprobes; both probes reacted with the corresponding gRNA (results not shown), suggesting generation of dsRNA forms of the GVA-GFP-260 minireplicon.

GVA-GFP-minireplicon-mediated RNA silencing

DsRNA is a well known inducer of the plant RNA silencing response, therefore, we intended to examine whether expression of such minireplicon in plants would trigger this defense mechanism. The cDNA of GVA-GFP-minireplicon was assembled into the binary vector to generate the designated clone pGVA-GFP-269 (Fig. 2A). *Agrobacterium* carrying pGVA-GFP-269 was then used to infiltrate leaves of *N. benthamiana* plants with, or without, the viral suppressor p10 of GVA. Expression of GFP in infiltrated leaves was analyzed with a confocal microscope and quantified by means of MICA analysis. The results, presented in Fig. 2B, show remarkable increases in green fluorescence levels in

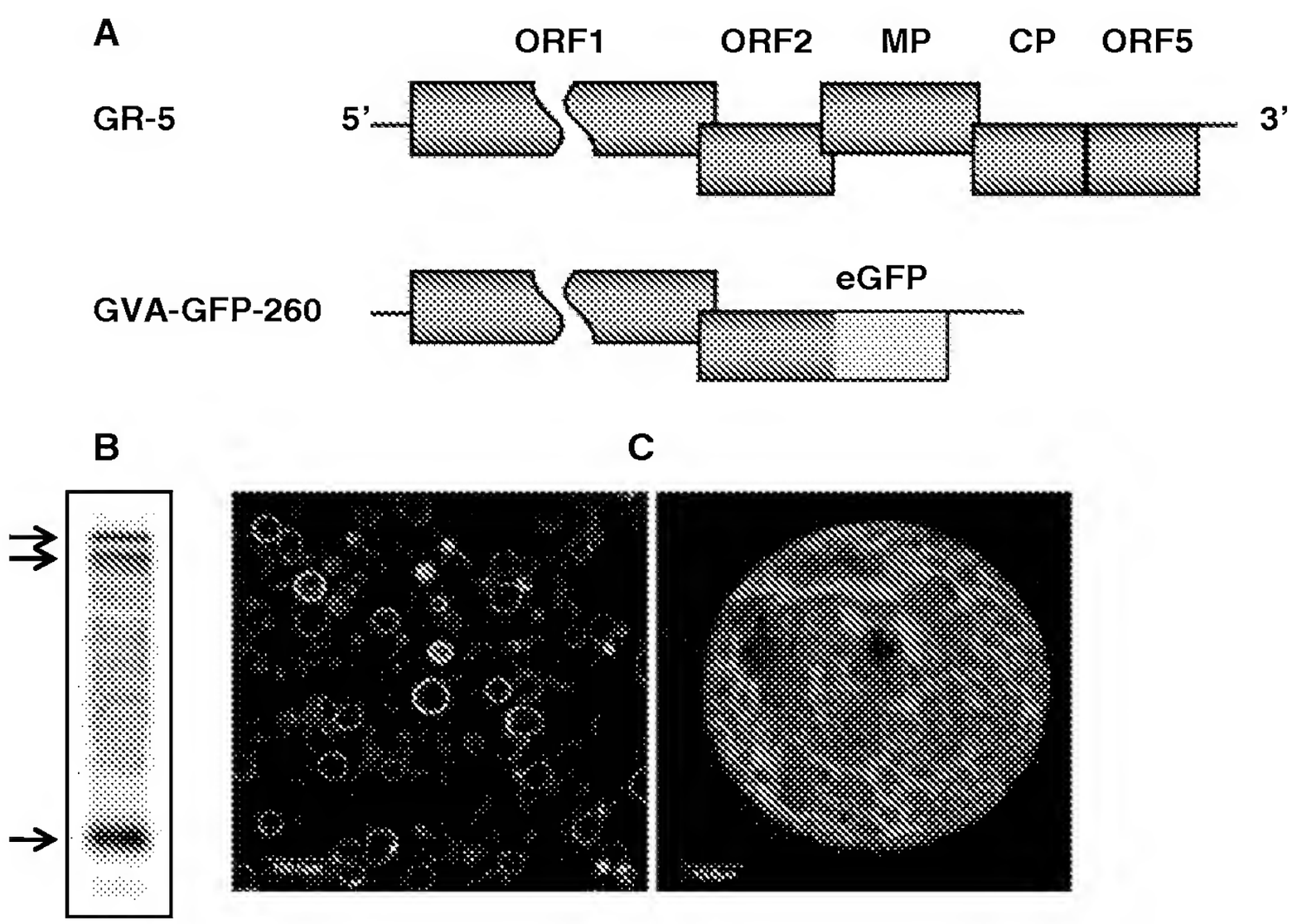


Fig. 1 Infectivity of the GVA-GFP-260 minireplicon in *Nicotiana benthamiana* protoplasts. **a** Schematic diagrams of the full-length infectious clone of GVA (GR5) and the GVA-GFP-260 minireplicon cloned into the pUC57. Boxes represent open reading frames (ORFs); CP, coat protein; MP, movement protein. **b** Northern blot analysis of RNA extracted from *N. benthamiana* protoplasts infected with in vitro-synthesized RNA transcribed from pGVA-GFP-260. The arrows point to GVA RNAs **c**. GFP expression in *N. benthamiana* protoplasts infected with GVA-GFP-260 (images with single and multiple protoplasts are shown). GFP fluorescence was monitored at 3 dpi by means of laser confocal microscopy

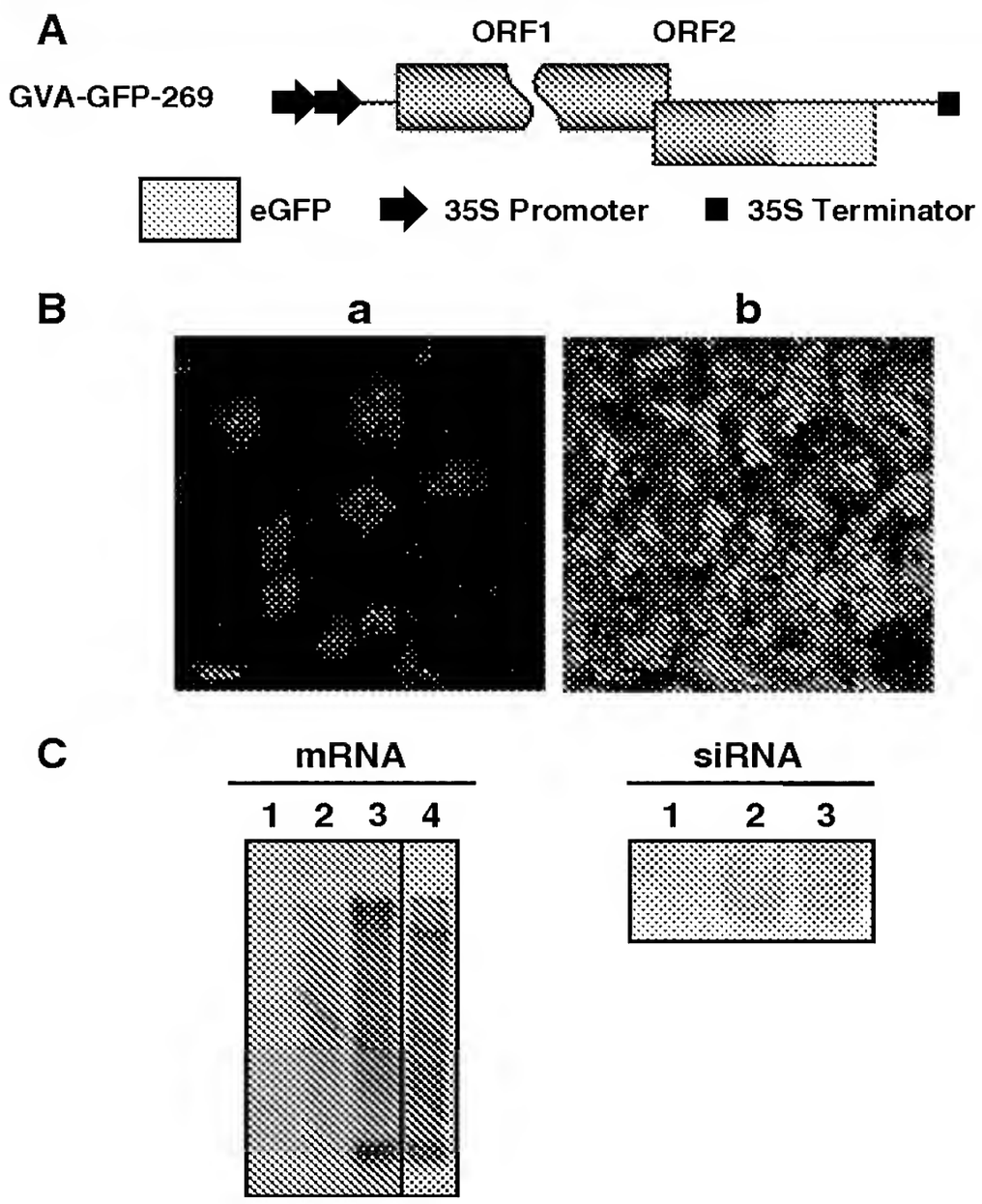


Fig. 2 Infectivity of the GVA-GFP-269 minireplicon in *N. benthamiana* plants. **A** Schematic diagram of the GVA-GFP-269 minireplicon cloned in the pCAMBIA vector. **B** Expression of GFP in *N. benthamiana* leaves agro-infiltrated with GVA-GFP-269 minireplicon (**a**), and with GVA-GFP-269 minireplicon plus GVA p10 (**b**). GFP fluorescence was monitored at 7 dpi by means of laser confocal microscopy. **C** Northern blot analyses for mRNA and siRNA extracted from *N. benthamiana* leaves non-infiltrated (lane 1), agro-infiltrated with GVA-GFP-269 minireplicon (lane 2), and agro-infiltrated with GVA-GFP-269 minireplicon plus GVA p10 (lane 3). The RNA in lane 4 was extracted from GVA-infected plants. The hybridization was conducted with a riboprobe specific to 5′-terminus of the GVA genome

leaves agro-infiltrated with pGVA-GFP-269 plus the GVA p10 suppressor, compared with the level in leaves infiltrated with only pGVA-GFP-269. Moreover, northern blot analyses, conducted for detection of mRNA and siRNA levels, revealed reduction of siRNA and increase of gRNA in leaves co-infiltrated with the GVA-GFP-269 and the GVA p10 suppressor compared with the levels in leaves infiltrated with GVA-GFP-269 alone (Fig. 2C, see also Fig. 3D lane mr). These results suggested that expression of the minireplicon GVA-GFP-269 induced RNA silencing in infiltrated leaves, and that expression of p10 suppressor counteracted this defense mechanism.

Fig. 3 Examination of transgenic *N. benthamiana* expressing the GVA-GFP-269 minireplicon. **A** PCR amplification, with the primer combination GVA10 and GVA125 (see the Experimental procedures section for sequence information), conducted for the genomic-DNA extracted from transgenic plants (lanes 1–10). Lanes 11 and 12 show PCR amplification for DNA extracted from a non-transgenic plant and a DNA of cloned GVA-GFP-269, which were used as negative and positive controls, respectively. Lane M shows DNA size markers **B** Southern blot analysis, with a riboprobe specific to the 5-terminus of the GVA genome, conducted for *Hin*dIII-digested gDNA extracted from transgenic plants (lanes 1–7) and from a non-transgenic plant (lane 8). Lane 9 included *Hin*dIII-digested DNA of pGVA-GFP-269. **C** northern blot analysis of transgene-derived RNA expression performed on 15 transgenic plants. Total RNA was extracted and hybridized with a riboprobe specific to the 5-terminus of the GVA genome. Lanes H and I included RNA extracted from healthy and GVA-infected non-transgenic *N. benthamiana* plants, respectively. **D** Hybridization analysis of siRNA molecules of transgenic plants expressing the GVA-GFP-269 minireplicon (lanes 1–6) with a riboprobe specific to the 5′-terminus of the GVA genome. Lanes H and mr contained RNA extracted from a healthy plant and from leaves agro-infiltrated with the GVA-GFP-269 minireplicon, respectively. **E** GFP expression in plantlets at early stages (**a**) and in mature plants (**b**). GFP fluorescence was monitored by means of laser confocal microscopy

Consistent expression of minireplicon in transgenic plants

As a result of our finding on the effectiveness of the GVA-GFP-minireplicon to induce the plant RNA silencing mechanism, we aimed to utilize this tool to develop resistance to GVA infection by producing transgenic plants with consistently activated RNA silencing. The designated clone pGVA-GFP-269 was used for *Agrobacterium*-mediated transformation of *N. benthamiana*. At least 36 lines of T0 plants that were confirmed as transgenic by GUS, PCR, and Southern blot assays, as well as by monitoring GFP expression (see below), were chosen for further production of T1 followed by T2 generations. All of the T0, T1, and T2 transgenic plants ($n > 400$) have maintained normal phenotypes throughout their growth period.

In order to confirm transformation of the GVA-GFP-269 transgene the kanamycin resistant and GUS positive plants were further tested by PCR amplification of an approximately 1.1-kb fragment comprising ORF2 and eGFP sequences, using the GVA14 and GVA125 primers. Out of every 10 transgenic plants, seven to nine were found to be positive in the PCR assay (analyses of some plants are presented in

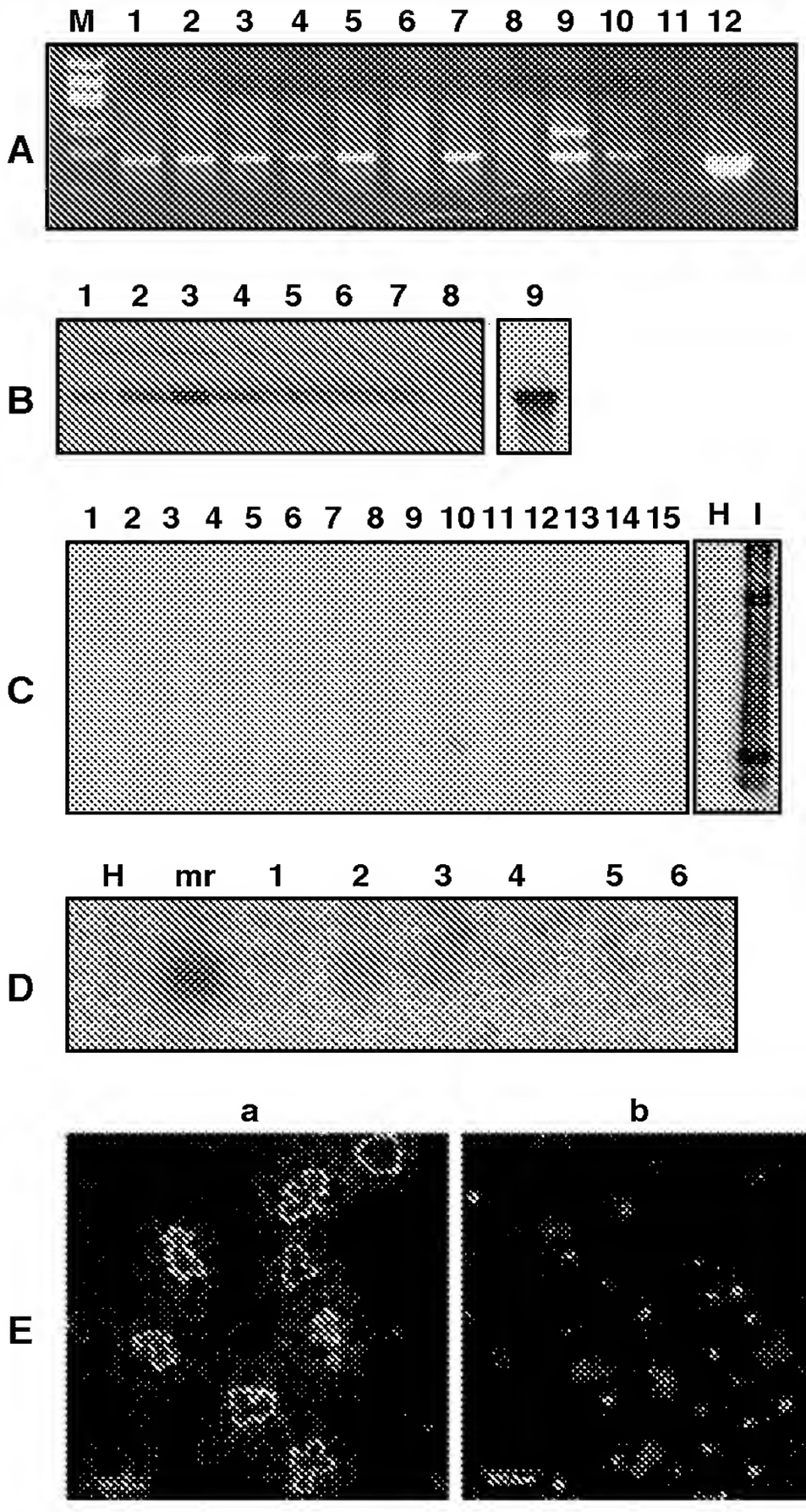


Fig. 3A). The DNA extracts of the positive plants were further examined by Southern blot analysis. The DNA was digested with *Hin*dIII and subjected to electrophoresis in agarose gel, followed by blotting onto membranes and hybridization with DIG-labeled riboprobe specific to the 5′-1.0 kb of the GVA genome. We found that this probe reacted in a specific manner with the DNA samples of all the transgenic plants that were positive in the PCR assay (analyses of some plants are presented in Fig. 3B).

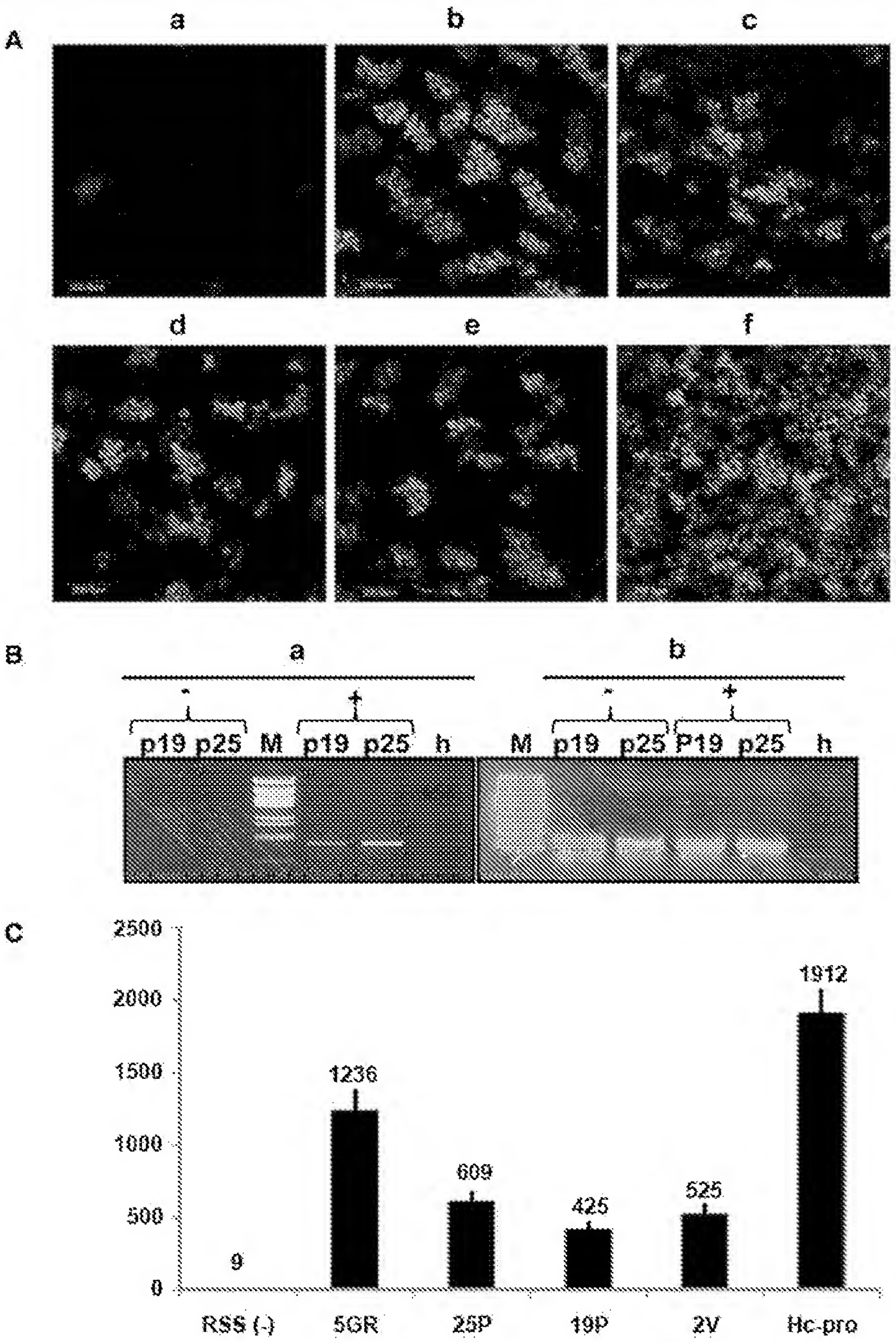
To examine production of mRNA corresponding to the transgene, total RNA from the transgenic plants was extracted and used for northern blot analysis, using a 5′-1-kb-specific GVA riboprobe. This analysis resulted in detection of remarkably low levels of mRNA corresponding to the transgene mini-GVA-GFP-269 (analyses of some plants are presented in Fig. 3C).

Since the GVA-GFP-269 was expected to result in GFP expression, transgenic plants were additionally analyzed by means of confocal microscopy. All transgenic plants, which were confirmed as transgenic by GUS, PCR, and Southern blot assays, showed extremely low green fluorescence. In general, GFP expression was observed in more cells of plantlets in the early stages following transformation than of mature plants (Fig. 3E-a). In leaves of mature T0, T1, and T2 plants, GFP expression was observed mainly in stomatal guard cells (Fig. 3E-b).

PTGS in transgenic plants

Detection of low levels of RNA corresponding to the transgene GVA-GFP-269 and the observation of low green fluorescence in leaves of transgenic plants would suggest that a strong plant response counteracted the transcribed transgene. To determine whether a plant RNA silencing response counteracted transgene transcription, leaves of transgenic plants were infiltrated with the potyvirus Hc-pro, which is known to suppress the plant RNA silencing mechanism. In comparison to non-infiltrated leaves of the transgenic plant, the Hc-pro-infiltrated leaves of these plants showed at least a 200-fold increase in green fluorescence, as measured and calculated by means of confocal microscopy and the MICA program (Fig. 4A-a, A-f, C). Similarly, infiltration of the GVA-GFP-269 transgenic plants with p10, p19, p25, and v2, which are other known viral silencing suppressors derived from GVA, *Tomato bushy stunt virus* (TBSV), PVX, and *Tomato yellow leaf curl virus* (TYLCV), respectively, resulted in significant increases in the green florescence levels, ranging from 47- to 137-fold (Fig. 4A-b to -f, C).

To further confirm the effects of viral suppressors on expression of the transgene, we performed RT-PCR analysis. Total RNA was extracted from transgenic plants infiltrated with the p25 or p19, and from non-infiltrated transgenic plants. The RNA was treated with DNase and used for cDNA synthesis using an oligonucleotide composed of poly (T). The cDNA was used for PCR with the primers GVA14 and GVA125 to amplify a 1.1-kbp fragment corresponding to GVA sequence. As a control, an ~100-nts fragment of the host Elongation Factor-1α (EF-1 α)



RNA was amplified by RT-PCR from the same cDNA reactions. Figure 4B shows amplification of GVA sequences from transgenic plants infiltrated with the p25 and p19 suppressors, but not from non-infiltrated transgenic plants. The cDNA of the host enhancer factor was similarly amplified from transgenic plants, infiltrated or not, with the virus suppressor (Fig. 4B). Taken together, these results suggest that the GVA-GFP-269 transgenic plants were silenced and that suppression of their activated PTGS response resulted in increased expression levels of the transgene GVA-GFP-269 minireplicon.

◀ **Fig. 4** Examination of the effect of suppressors of RNA silencing on the transgene expression. **A** Representative confocal images of leaves of GVA-GFP-269 transgenic plants agro-infiltrated with the suppressors of RNA silencing GVA p10 (**b**), TBSV p19 (**c**), PVX p25 (**d**), TYLCV V2 (**e**), and PVY Hc-pro (**f**). (**a**) shows a confocal image of a non-infiltrated leaf of a GVA-GFP-269 transgenic plant. All images were taken at 5 dpi. **B** RT-PCR amplification of a 1.1-kb transgene-specific fragment (**a**) and, as a control, an approximately 100-nts fragment of a host elongation factor-1 α (GenBank accession number X14449) (**b**), performed on RNA extracted from GVA-GFP-269 transgenic plants infiltrated (+) or non-infiltrated (−) with the suppressors of RNA silencing p19 and p25. h; represents a negative control performed for a DNA-free PCR. Lanes M show DNA size markers. **C** GFP quantification, by MICA image analysis, in leaves of GVA-GFP-269 transgenic plant which were agro-infiltrated with different suppressors of RNA silencing. (−) RSS; represents GFP quantification in a non-infiltrated leaf of a GVA-GFP-269 transgenic plant. Fluorescence intensity per square micrometer was evaluated with MICA image analysis software (Cytoview, Petach Tikva, Israel) and the Microsoft Excel software (Microsoft, Redmond, WA). Values shown are the averages of quantitative analyses obtained from five different regions of infiltrated leaves. Error bars represent standard errors of the means

Virus resistance

The results described above show that the GVA-GFP-269 transgenic plants displayed phenotypes that we attribute to an activated PTGS response. Therefore, we wanted to examine whether these transgenic plants would resist invasion of the wild-type GVA. Transgenic and non-transgenic *N. benthamiana* plants were inoculated with sap extracted from GVA-infected *N. benthamiana* plants. On the non-transgenic plants, typical systemic GVA symptoms were observed on newly generated leaves at 6–8 dpi; they appeared as vein clearing, leaf curling, and mottling (Fig. 5a). However, 60% of T1 transgenic plants were symptomless throughout the time they could be maintained (Fig. 5b); in these plants, no accumulation of GVA was detected by means of northern blot and PCR analyses (results not shown). Examination of the T2 progeny plants, which were obtained from selected resistant plants, revealed 90–95% of the plants to be symptom free, and free of GVA accumulation and 5–10% of plants with notably moderate and delayed symptoms. These observations suggest that the transgenic plants expressing the GVA-GFP-269 minireplicon exhibited resistance to GVA infection. Similar features of partial resistance exhibited by T1 and T2 plants have been reported on other systems (Gal-On et al. 1998), though the reason for this is not known.

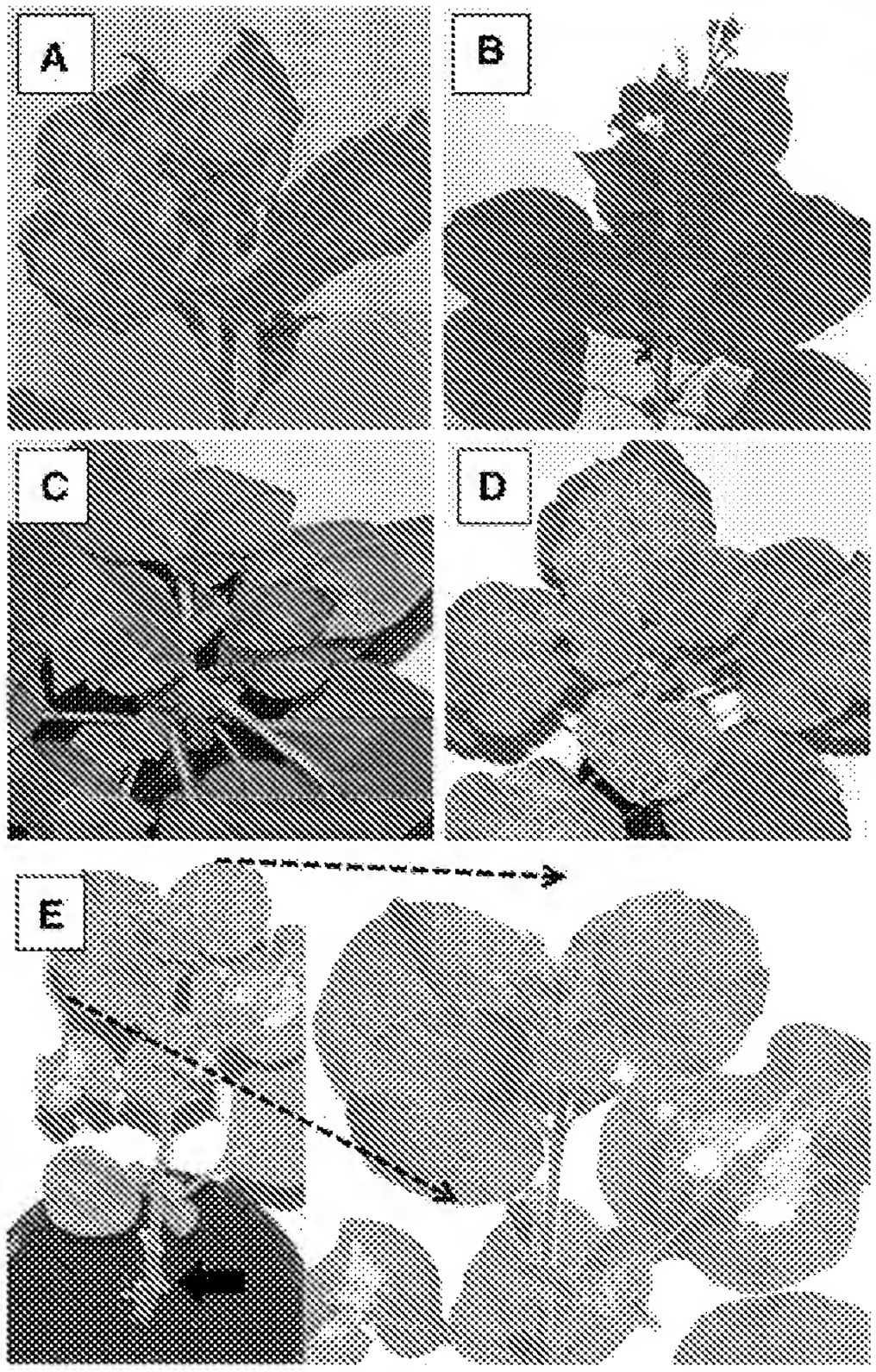


Fig. 5 Phenotypes on *N. benthamiana*. **a** symptoms on a GVA-inoculated non-transgenic *N. benthamiana*. **b** A non-symptomatic GVA-GFP-269 transgenic plant inoculated with GVA. **c** and **d** Symptoms on GVA-GFP-269 transgenic plants inoculated with PVY and GVB, respectively. **e** A non-symptomatic graft (non-silenced *N. benthamiana* grafted onto a silenced GVA-GFP-269 plant) inoculated with GVA. A higher magnification image is shown on the right

Since RNA silencing is a sequence specific process; it is likely that the GVA-GFP-269 transgenic plants exhibit resistance only for GVA and not for other viruses. To examine this possibility, transgenic plants were inoculated with a sap extracted from PVY- or GVB-infected *N. benthamiana* plants. Both transgenic- and non-transgenic plants were found to be susceptible to these viruses; systemic symptoms of virus infection appeared on newly generated leaves at 6–12 dpi (Fig. 5c, d). This suggested that the

GVA-GFP-269 transgenic plants exhibited a GVA-specific PTGS-involved resistance.

Systemic spread of virus resistance

Transmission of silencing signals from silenced rootstocks to non-silenced scions is a well known phenomenon and has been reported in several studies (Palauqui et al. 1997; Voinnet et al. 1998). To investigate whether the GVA-GFP-269-silenced transgenic rootstocks could induce RNA silencing in grafted non-silenced scions, non-transgenic plants were top-grafted onto GVA-GFP-269 transgenic plants. The newly developed leaves that appeared on the non-transgenic scions were inoculated with a GVA-containing sap extract. For control, non-transgenic scions were similarly grafted onto non-transgenic plants, and similarly inoculated. The inoculated plants were monitored for development of symptoms while they were maintained in the greenhouse. Whereas the control plants developed symptoms of GVA infection at 6–10 dpi (data not shown), 70–90% of grafted T2 progeny plants were found to be symptomless (Fig. 5e). Similar findings were obtained from five independent experiments. In addition to monitoring symptom appearance, the inoculated grafted scions were examined for GVA infection by means of northern blotting and RT-PCR amplification. No hybridization signals or PCR products, which could suggest GVA infection, were obtained in any of the symptomless grafted scions (results not shown). These results indicated that GVA-minireplicon-mediated RNA silencing was active in the non-transgenic scions through the action of the silenced transgenic rootstocks.

Infection of silenced mini-GVA-GFP-269 transgenic plants with viruses other than GVA

The GVA-resistant plants, transformed with the GVA-GFP-269, were not protected against PVY or GVB infection, which would suggest that these viruses counteracted the plant PTGS response. On the basis of this interpretation, one would expect that infection of the transgenic plants with viruses other than GVA was likely to result in increased expression of the GVA-GFP-269 transgene. To examine this possibility, we used confocal microscopy and northern blot analyses to examine GVA-GFP-269 transgenic plants that had been inoculated with PVY or GVB and that showed symptoms of virus infection. The results presented in Fig. 6 show that the levels of green fluorescence, as well as the RNA accumulation by the transgene, were extensively higher in the PVY- or GVB-infected transgenic plants, than in non-inoculated transgenic plants. These findings indicate that expression of the GVA-GFP-269 transgene was increased because of PVY or GVB infection. Thus, we concluded that infection of the GVA-GFP-269-silenced plants with viruses other than GVA could cause suppression of their consistently activated PTGS response.

Since GVB infection appeared to affect suppression of PTGS in the GVA-GFP-269 transgenic plants, we aimed to examine whether GVB-inoculated transgenic plants still displayed GVA resistance. A mixture of two sap extracts, prepared from GVA- and GVB-infected plants, respectively, was used to inoculate silenced GVA-GFP-269-transgenic plants, and systemic symptoms of virus infection appeared on newly generated leaves at 6–12 dpi. To determine which virus caused the infection, RT-PCR analyses were conducted to amplify a 360-nts fragment of GVB ORF5 and a 300-nts fragment of GVA ORF4, which is not a part of the transgene GVA-GFP-269. The results presented in Fig. 6C indicate that both infections—GVB and GVA—had been established in the inoculated plants. Similarly, we found that inoculating the GVA-GFP-269 transgenic plants with a mixture of PVY and GVA resulted in accumulation of GVA and PVY (results not shown). As expected with the control plants, when a sap containing a single virus—GVB or GVA—was used the inoculated transgenic plants, were found to be susceptible to GVB (Fig. 6C-a lane 1), but resistant to GVA (Fig. 6C, lane 6). In light of these results, we concluded that infection of the silenced GVA-GFP-269 transgenic plants with a virus other than GVA could cause suppression of the resistance system that was activated for GVA infection.

Discussion

We have demonstrated here that: (i) a GVA minireplicon can mediate gene silencing in *N. benthamiana* plants; (ii) transgenic plants expressing the GVA-minireplicon exhibited virus resistance

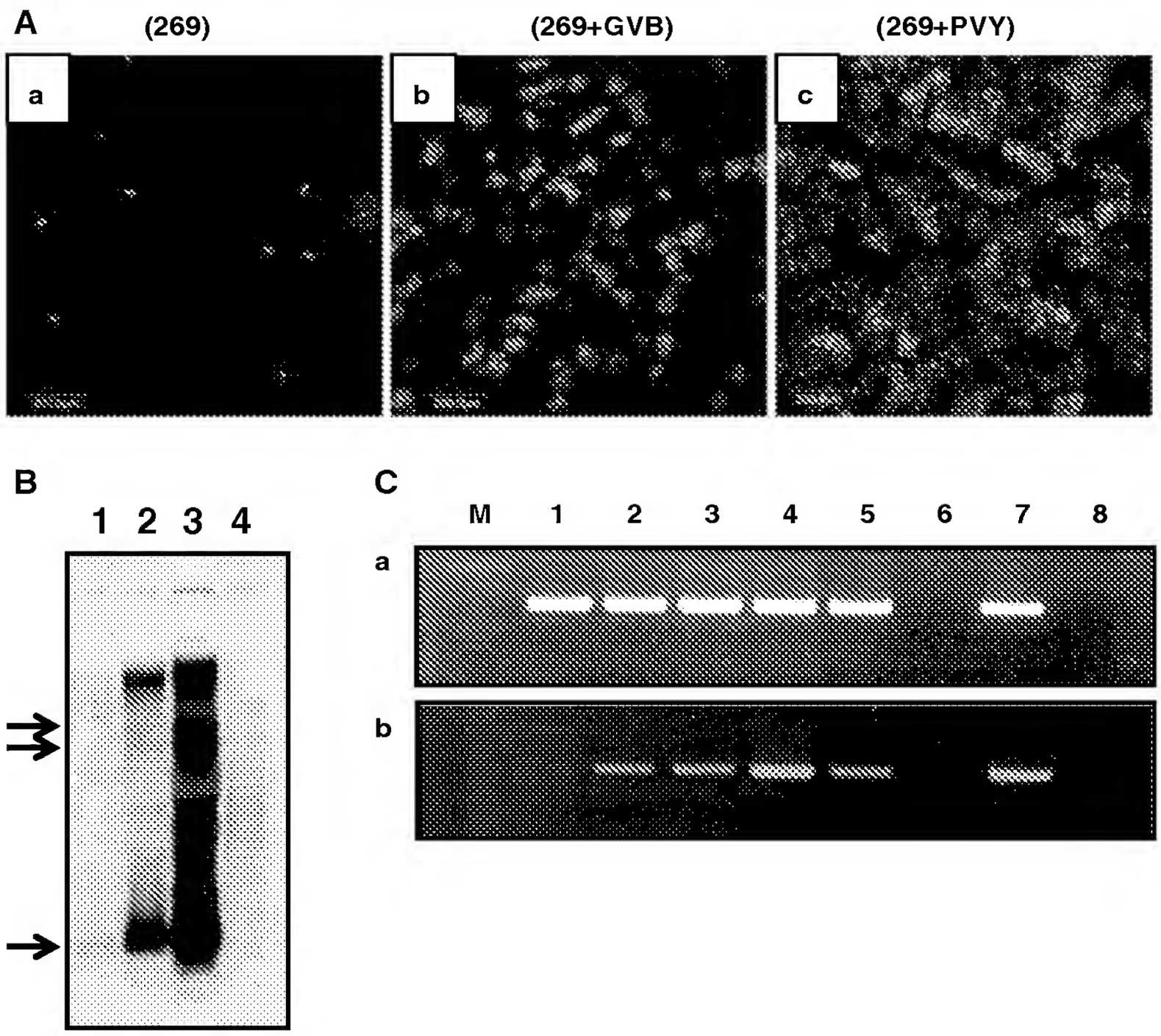


Fig. 6 Effect of GVB infection on GVA-GFP-269 transgenic plants. **A** Representative confocal images of GVA-GFP-269 transgenic plants, non-inoculated (**a**), and GVB- (**b**) or PVY- (**c**) inoculated. **B** Northern blot analysis of transgene expression in GVA-GFP-269 transgenic plants, non-inoculated (lane 1), and GVB- (lane 2) or PVY- (lane 3) inoculated. Lane 4 contains RNA extracted from a non-transgenic *N. benthamiana* plant. Arrows indicate GVA RNAs which accumulated because of replication of the transgene. **C** RT-PCR analyses, which amplified a 360-nts fragment of GVB ORF5 (**a**) and a 300-nts fragment of GVA ORF4 (**b**), performed on RNA extracted from GVA-GFP-269 transgenic plants inoculated with GVB (lane 1), GVB plus GVA (lanes 2–5), or GVA (lane 6). Lane 7; RT-PCR amplification of RNA extracted from non-transgenic *N. benthamiana* plant inoculated with GVB or GVA. Lane 8; RT-PCR performed on RNA extracted from non-transgenic *N. benthamiana*. Lane M shows DNA size markers

specifically targeted against GVA; and (iii) minirepl-icon-mediated resistance was transmissible to non-transgenic scions grafted onto transgenic rootstocks.

Virus-based minireplicon systems, and their use for induction of plant PTGS response, can serve as useful tools in both research and applied biotechnology. Chiba et al. (2006) described an agroinfection assay that used *Beet yellow virus* (BYV)-based minireplicons for identification of viral suppressors of RNA silencing. The GFP-tagged GVA-minirepl-icon used in the present study, contains the sequences of the 5′ UTR, ORF1, ORF2 with the eGFP gene fused to its 3′ end, and the 3′ 290 nts (Fig. 1A). The ORF2 gene is dispensable in RNA replication (Galiakparov et al. 2003a, c); however, because its N-terminus overlaps with the C-terminus of the RdRp, we chose to include it in the minireplicon sequence.

The GVA-GFP minireplicon was found to be an efficient mediator of PTGS induction. There are two features of this minireplicon that are important for the plant PTGS response. First, there is the ability to replicate and to express ORF2-GFP fused product via the ORF2 internal promoter (Galiakparov et al. 2003a). This is associated, similarly to most, if not all, replicating viral RNAs, with generation of dsRNA molecules, which are well known as potent activators of PTGS (Covey et al. 1997; Ruiz et al. 1998; Baulcombe 1999). Continuous replication and amplification of the minireplicon would, therefore,

allow consistent production of the PTGS inducer—dsRNA. The second feature of the GVA-minireplicon is the absence of the virus-encoded suppressor p10. Obviously, if the GVA minireplicon encoded a viral suppressor of silencing, it would compromise the PTGS response. Moreover, the GVA-minireplicon lacks the ORFs that encode the MP and the CP, which indicates that these genes are dispensable for induction and spread of PTGS within the plant.

The GVA minireplicon was found to be an efficient tool for activating PTGS in infiltrated *N. benthamiana* leaves. In light of this, we aimed to utilize it for developing transgenic plants resistant to GVA infection. We produced transgenic *N. benthamiana* plants expressing the GVA minireplicon, which were found to be useful: (i) as GVA-resistant plants; and (ii) as a tool for analyzing activities of viral suppressors of RNA silencing. Expression of the GVA minireplicon in infiltrated leaves or in transgenic plants resulted in low levels of green fluorescence. To determine whether the low level of GFP expression was due to PTGS or to inefficient replication of the GVA minireplicon, we exploited a viral suppressor of PTGS. If there had been inefficient replication, the introduction of a viral suppressor of PTGS into the infiltrated leaves or into the transgenic plants would not have affected GFP expression from the GVA minireplicon. However, if PTGS had been activated, the expression of the GVA minireplicon and, consequently, the green fluorescence level would have increased in the presence of the viral suppressor of PTGS. The experiments that have been conducted indicated clearly that the green fluorescence levels increased remarkably because of expression of the viral suppressor of PTGS. Moreover, GVB- or PVY-mediated increase of GFP expression in transgenic plants also suggested that these viruses, too, suppressed PTGS. Currently, the GVA-minireplicon is routinely used in our laboratory as a tool to examine and identify PTGS suppressors of various grapevine viruses.

Expression of GFP varied in plant leaves in the course of development of mature transgenic plants; GFP expression was detectable in more cells in young T0 plantlets than in mature and old transgenic plants. This observation suggested that the PTGS response was induced in more cells of mature leaves than of young leaves on transgenic plants, probably because of the spread of PTGS. In mature leaves of transgenic plants, GFP expression was detectable mostly in stomatal guard cells, which would suggest ineffective spread of the induced PTGS signal into stomatal guard cells, probably because of their being symplastically isolated. Similar observations of inefficient PTGS spread into stomatal guard cells were reported previously (Voinnet et al. 1998; Mlotshwa et al. 2002).

Most of the plants that were confirmed to be transgenic by Southern blot, RT-PCR and GFP expression, showed extreme GVA resistant, though some (5–10% of T2 progeny) exhibited delayed, mild symptoms of virus infection. Northern blot analysis of the latter revealed extremely low levels of the minireplicon transgene expression, which suggests that PTGS did not result in the complete suppression of the target RNAs. Consequently, these plants would exhibit consistently activated PTGS, which can specifically target GVA and prevent its invasion for as long as the plant could be maintained. These findings confirm what is already known about the inverse correlation between the transcriptional level of the introduced virus-derived sequence and the degree of virus protection (Mueller et al. 1995; Prins et al. 1996).

The grafting experiments indicated that transmission of the transgene-derived resistance signal from transgenic *N. benthamiana* plants to non-transgenic plants had occurred. We found that non-transgenic plants grafted onto transgenic plants expressing the GVA minireplicon became resistant to GVA infection with more than 70–90% efficiency. In contrast, however, 100% of control grafts onto non-transgenic plants showed GVA infection. These findings suggest transmission and movement of a transgene-specific PTGS signal into distant tissues. The mechanism of and requirements for the systemic transmission of the RNA silencing signal are not well understood; however, the transgene-derived siRNA molecules are thought to play an important role in the transmission throughout the plant tissues (Palauqui et al. 1997; Voinnet et al. 1998). Expression of replicating viral RNAs in transgenic scions, which would result in amplification of the transgene-specific PTGS signals, could be an important requirement in establishing efficient GVA silencing in non-transgenic scions in most grafts. However, we assume that the amount of the transgene-derived siRNAs produced in the transgenic scions was not sufficient to overcome suppression of PTGS by GVA in susceptible grafts (10–30%).

Since GVA resistance in the transgenic plants appeared to be derived from transgene-specific PTGS, one would expect that infection of these plants by a virus other than GVA (with suppression of RNA-silencing activity) should affect the phenotype GVA resistance. Naturally, GVA infection in grapevine plants frequently is associated with infection with another virus such as GVB (Shi et al. 2004); GVA and GVB belong to the same *Vitivirus* genus and share 53% sequence identity. Transgenic plants that express the GVA-minireplicon and exhibit GVA resistance were found to be susceptible to GVB infection, although the virus infection symptoms were milder and appeared 5–7 days later than in the control GVB-infected non-transgenic plants. Similarly to GVA, the GVB genome is thought to encode a suppressor of RNA silencing. GVB infection resulted in increased accumulation of the transgene minireplicon RNA and, consequently, increased GFP expression, which suggests suppression of the transgene-specific PTGS. Thus, GVB infection, as well as infection by other viruses such as the distinct virus PVY, would be expected to break down resistance to GVA in transgenic plants. The experiments we conducted, in which a sap containing a mixture of GVA with GVB or GVA with PVY was used for inoculation of the transgenic plants, confirmed this notion by demonstrating GVA accumulation and symptoms of virus infection in these plants.

Grapevine is considered one of the most economically important fruit crops worldwide, and grapevine viruses are the major causal agents of diseases in vineyards worldwide (Martelli 1993; Martelli et al. 1997). Development of genetically engineered plants that exhibit virus resistance could be a promising strategy for controlling or reducing the damage caused by viruses. Our present study indicated the potential utility of the GVA-minireplicon as the basis of a strategy for gene silencing in *N. benthamiana* transgenic plants. However, in imparting virus resistance based on transgene-specific PTGS it is necessary to take into account the possibility of infection by other viruses, which might cause suppression of the induced transgene-specific PTGS, as shown in the present study. Currently, we have under development, minireplicon-based transgenic plants expressing PTGS-involved resistance specific to multiple grapevine viruses.

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